

Regional IFN γ expression is insufficient for efficacious control of food-borne bacterial pathogens at the gut epithelial barrier

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Abstract

IFN γ is critical for host defence against various food-borne pathogens including *Salmonella enterica* and *Listeria monocytogenes*, the causative agents of salmonellosis and listeriosis, respectively. We investigated the impact of regional IFN γ expression at the intestinal epithelial barrier on host invasion by salmonellae and listeriae following oral challenge. Transgenic mice (IFN γ -gut), generated on an IFN γ knock-out (KO) background, selectively expressed IFN γ in the gut driven by the modified liver fatty acid-binding protein (Fabp)^{4 \times at -132} promoter. Infections with attenuated *S. enterica* Typhimurium or with *L. monocytogenes* did not differ significantly in IFN γ -KO, IFN γ -gut and wild-type mice. Further, *Listeria*-specific CD4⁺ and CD8⁺ T cells were not altered in IFN γ -gut mice. Thus, this model indicates that local IFN γ expression by non-immunological cells in the distal part of the small intestine, caecum and colon is insufficient for prevention of gut penetration by *S. enterica* Typhimurium and *L. monocytogenes*.

Introduction

Salmonella enterica and *Listeria monocytogenes* are intracellular bacterial pathogens, which enter the host through the gut epithelial barrier during natural infection (1, 2). Hence, the intestinal epithelium provides a first line of defence against these food-borne pathogens. Once these pathogens have successfully penetrated the epithelium, they rapidly spread to other tissue sites. Following oral infection, salmonellae replicate in mucosa-associated lymphoid tissues such as the Peyer's patches (PP) and subsequently disseminate via the mesenteric lymph nodes (MLNs) (3). *Listeriae* and salmonellae, in addition to invading the host via PP in the small intestine and disseminating via MLN, can enter through the colon (4) or via CD18-expressing phagocytes (5), respectively. *Listeriae* that enter through the colon subsequently spread via the caudal lymph node (CLN) (4, 6). Innate immune defence against infectious agents is mediated by various cytokines, which interact in a highly regulated network (7, 8). The importance of IFN γ in in-

testinal host defence against *S. enterica* Typhimurium and *L. monocytogenes* has been extensively documented (3, 9–16). This is true both for systemic and regional defence. Previous studies identified elevated levels of IFN γ mRNA in gut-associated lymphoid tissue after challenge with *S. enterica* Typhimurium (15). In PP, IFN γ mRNA was detected at 6 h, in MLN at 24 h and in spleen at 4 days after challenge. Administration of neutralizing mAbs to IFN γ completely abrogates resistance to oral challenge with salmonellae and listeriae (9, 15) and susceptibility to systemic infection is greatly enhanced (17). IFN γ is produced by both activated T cells and NK cells during host defence against these pathogens (7, 16). In addition, impaired host intestinal immunity in IFN γ -deficient mice following *S. enterica* Typhimurium or *L. monocytogenes* oral challenge has been demonstrated (3, 18, 19).

We generated IFN γ transgenic mice, which exclusively produce IFN γ in the gut on a general IFN γ knock-out (KO)

background (IFN γ -gut) to directly examine the role of regional IFN γ production in the intestinal epithelium against the intracellular pathogens *S. enterica* Typhimurium and *L. monocytogenes*. The expression of IFN γ is driven by the modified liver fatty acid-binding protein (Fabpl^{4 \times} at -132) promoter that exhibits gut-specific expression with increasing activity from the proximal to the distal part of the small intestine as well as in the caecum and colon (20). A low level of expression in the kidney was also reported (20). The IFN γ -gut mice were orally infected with *S. enterica* Typhimurium, strain SL7207, which is deficient in *aroA* or with *L. monocytogenes* strain EGD, and penetration of the intestinal epithelial barrier by these pathogens was analysed. Moreover, we characterized T cell responses in the different mouse strains following infection with *L. monocytogenes*. Our experiments reveal that despite similar pathogen-specific CD4⁺ and CD8⁺ T cell responses in IFN γ -gut mice, regional IFN γ expression by epithelial cells is insufficient for efficacious control of infection.

Methods

Generation of IFN γ -gut mice

The Fabpl^{4 \times} at -132 promoter was cleaved from a recombinant plasmid (kind gift from J. Gordon, St Louis, Missouri, USA) using *EcoRI* and *BamHI* restriction enzymes. A 469-bp IFN γ cDNA fragment was PCR amplified from a recombinant plasmid containing the full-length IFN γ cDNA with oligonucleotides containing *BamHI* and *XbaI* restriction sites. The 5'-end primer was 5'-CGGGATCCATGAACGCTACACACTGCATC-3' and the 3'-end primer was 5'-GCTCTAGATCAGCAGCGACTCCTTTTCCGC-3'. The resulting PCR product was cleaved with *BamHI* and *XbaI* at the 5'- and 3'-ends, respectively. The SV40 small t-intron/polyadenylation sequence was PCR amplified from the vector pGL2-Basic (Promega, Madison, WI, USA) with oligonucleotides containing restriction sites that enabled the resulting product to be cleaved with *XbaI* and *NotI* at the 5'- and 3'-ends, respectively. The Fabpl^{4 \times} at -132 promoter, the IFN γ cDNA and the SV40 small t-intron/polyadenylation sequence were cloned into pBluescript in that order. Transient transfections using lipofectin (Invitrogen, Carlsbad, CA, USA) with this recombinant plasmid into 293T cells were performed according to the manufacturer's protocol and supernatants removed and concentrated 4-fold using AMICON (10 000 kDa cut-off) spin columns. IFN γ in supernatants was detected by ELISA using mAb R4-6A2 to capture the IFN γ and mAb XMG1.2 biotin labelled followed by streptavidin conjugated with peroxidase to detect the captured IFN γ . To generate the IFN γ -gut mice, the complete construct was cleaved out of the vector with *EcoRI* and *NotI*, gel purified and used for pro-nuclear injection of oocytes from an IFN γ -KO mouse on a C57Bl/6 background (kind gift from T. Stewart, South San Francisco, CA, USA).

RNA isolation and quantitative PCR

Mice were sacrificed before or after *Salmonella* or *Listeria* infection (see below). RNA from tissue samples (2 cm intestinal sections and ~50 mg from other organs) was isolated

with TRIzol (Invitrogen) using the FastRNA tubes green (Q-Biogene, Irvine, CA, USA) and subjected to DNase I treatment according to their protocol. Quantity and quality of isolated RNA were determined by the Bioanalyser and by OD measurement. Reverse transcription was performed with random primers using the Superscript reverse transcription kit from Invitrogen. Quantitative PCR was performed with SyBR Green mix (Applied Biosystems, Foster City, CA, USA) using the Applied Biosystems machine. The real-time PCR mixture was prepared as follows: 15 μ l SyBR Green mix, 0.15 μ l of a 100 pm solution each of forward and reverse primer and 5 μ l of a 1:15 diluted cDNA synthesized according to the reverse transcription kit. Each sample for real-time PCR was made in triplicate and mean of the resulting three values were taken. The following primers for IFN γ , IFN γ -inducible protein-10 (IP10) and IFN-inducible GTPase (IIGP) amplifications were used: IFN γ forward 5'-ACGGCACAGT-CATTGAAAGCCTA-3' and reverse 5'-GTCACCATCCTTTT-GCCAGTTCC-3', IP10 forward 5'-CCGTCATTTTCTGCCTC-ATCCT-3' and reverse 5'-GCTTCCCTATGGCCCTCATTCT-3' and IIGP forward 5'-GCCACCAATCTTCTGCTCTCTAAC-3' and reverse 5'-CTTCCAGCCAAATCTCTGCTTC-3'.

Histology

Macroscopic and microscopic examination of histopathology (inflammation) was performed *in situ* and in formalin-fixed intestinal tissue, respectively. Microscopical analysis was performed in a blind fashion for signs of infiltration, ulceration, mucosal thickening, haemorrhage and epithelial cell integrity.

Oral infections with salmonellae and listeriae

A frozen aliquot of deficient (*aroA*⁻) *S. enterica* Typhimurium (SL7207) or wild-type (wt) *S. enterica* Typhimurium (SL1344) was inoculated in 100 ml of Luria-Bertani (LB) medium containing 0.3 M NaCl and left overnight in a 37°C incubator. A frozen aliquot of *L. monocytogenes* strain EGD was inoculated in 100 ml tryptic soy broth and incubated at 37°C overnight with gentle shaking at 90 r.p.m. Bacterial cultures were harvested by centrifugation at 3000 \times g for 10 min in 50 ml Falcon tubes and washed twice in PBS. After OD₆₀₀ measurement, they were diluted with PBS from 0.5 \times 10⁹ to 1.5 \times 10¹⁰ colony-forming units (CFU) per ml (taking OD₆₀₀ as 10⁹ CFU) and 200 μ l was used for intragastric gavage. CFU were always controlled by plating of serial dilutions of the inoculum. To determine bacterial burdens, mice were sacrificed after 1 or 2 days, spleen and MLNs homogenized in PBS and serial dilutions of homogenates were plated on LB agar plates. Colonies were counted after overnight incubation at 37°C.

Purification of cells and intracellular cytokine staining

Lymphocytes from spleen, MLNs, small intestine epithelium and small intestine lamina propria were isolated as previously described (21, 22). Cells (1 \times 10⁶–4 \times 10⁶) were cultured in a 1-ml volume of RPMI 1640 medium supplemented with glutamine, Na-pyruvate, β -mercaptoethanol, penicillin, streptomycin and 10% heat-inactivated FCS. Cells were stimulated for 5 h with 10⁻⁶ M of the peptides listeriolysin O

amino acids 190–201 (LLO_{190–201}, NEKYAQAYPNVS) or ovalbumin_{257–264} (OVA_{257–264}, SIINFEKL). During the final 4 h of culture, 10 $\mu\text{g ml}^{-1}$ brefeldin A (Sigma Aldrich, Taufkirchen, Germany) was added. Cultured cells were washed and incubated for 10 min with rat serum and anti-CD16/CD32 mAb to block non-specific antibody binding. Subsequently, cells were either stained with PE-conjugated anti-CD4 mAb or PE-Cy7-conjugated anti-CD8 α mAb, and after 30 min on ice, cells were washed with PBS and fixed for 20 min at room temperature with PBS 4% PFA. Cells were washed with PBS and 0.1% BSA, permeabilized with PBS, 0.1% BSA and 0.5% saponin (Sigma) and incubated in this buffer with rat serum and anti-CD16/CD32 mAb. After 5 min, Cy5-conjugated anti-tumour necrosis factor α (TNF α) mAb was added. After a further 20 min at room temperature, cells were washed with PBS and fixed with PBS 1% PFA. Cells were analysed using a FACS Canto and DIVA software (Becton-Dickinson, Mountain View, CA, USA).

Antibodies

Anti-CD16/CD32 mAb (clone: 2.4G2) and anti-TNF α mAb (XT22) were purified from rat serum or hybridoma supernatants with protein G sepharose. Antibodies were Cy5-conjugated according to the standard protocols. PE-conjugated anti-CD4 mAb (GK1.5) and PE-Cy7-conjugated anti-CD8 α mAb (53-6.7) were purchased from BD PharMingen (San Diego, CA, USA).

Infection of mice for determination of T cell responses

C57Bl/6 mice were bred in our facility and experiments were conducted according to the German animal protection laws. All mice were infected with a recombinant *Listeria monocytogenes* strain-expressing ovalbumin (23), termed LmOVA. Mice were infected with 2×10^9 LmOVA by gastric incubation and were analysed on day 9 post-infection (p.i.).

Results

IFN γ expression driven by the modified *FabpI* promoter, *FabpI*^{4 \times} at -132

The transgenic construct contained mouse IFN γ cDNA downstream of the *FabpI*^{4 \times} at -132 promoter. To avoid any endogenous post-transcriptional regulatory sequences in

the IFN γ mRNA, the cloned 469-bp IFN γ cDNA contained only the sequence from the AUG start codon to the UGA stop codon. 293T kidney cells were transfected with the transgenic construct and production of IFN γ was determined. In the supernatant of transfected 293T kidney cells, but not of control cells, IFN γ was detected by ELISA (data not shown) indicating that the construct was functional.

Gut-specific and functional IFN γ expression in transgenic mice

The IFN γ -gut mice were generated on an IFN γ -KO background. In IFN γ -gut mice, the IFN γ cDNA signal was detected and mice were deficient for the endogenous IFN γ gene as determined by the PCR on DNA isolated from tail clips (data not shown). Compared with wt mice, the transgenic mice expressed IFN γ in the distal part of the small intestine at levels similar to wt mice and expression increased towards the caecum with a maximum level of expression in the colon. Quantitative PCR analysis (Table 1) revealed that IFN γ expression was elevated 19-fold in the caecum and 44-fold in the colon relative to IFN γ expression levels in wt mice. We analysed RNA levels of the IFN γ -responsive genes IP10 and IIGP to verify whether the transgene-encoded IFN γ was functionally active. Compared with wt mice, IP10 mRNA expression in IFN γ -gut mice was 2.5-fold and 5.3-fold higher in caecum and colon, respectively. IIGP RNA was 8-fold and 13-fold higher in IFN γ -gut caecum and colon, respectively (Table 1). There was an increase in IFN γ expression levels in wt caecum and colon, 48 h p.i. with *S. enterica* Typhimurium *aroA*⁻, as the IFN γ levels in both caecum and colon in IFN γ -gut mice were only ~9- and 8-fold higher, respectively, compared with wt mice (Table 1). Levels of IFN γ -responsive genes, IP10 and IIGP, were also up-regulated. IP10 levels were higher in caecum and colon of infected wt mice; in IFN γ -gut mice, levels were only -1.25-fold and +1.15-fold different while they were 2.54-fold and 5.35-fold higher when compared with naive wt mice. (A negative value such as -1.25-fold in IFN γ -gut mice versus infected wt mice indicates it is 1.25-fold greater in infected wt compared with IFN γ -gut mice.) Similarly, IIGP levels were only 3.6-fold and 2.3-fold higher in caecum and colon of IFN γ -gut mice compared with infected wt mice while these were 8-fold and 13-fold higher when compared with naive wt mice. As

Table 1. Expression levels of IFN γ , IP10 and IIGP in the small intestine, caecum and colon of IFN γ -gut mice relative to wt mice as estimated by quantitative PCR^a

Comparison of IFN γ -gut mice versus	Gene product	Expression level (fold difference) in			
		Small intestine (proximal region)	Small intestine (distal region)	Caecum	Colon
wt-naive mice	IFN γ	-35.75 \pm 0.30	-1.89 \pm 0.30	19.20 \pm 0.22	44.94 \pm 0.21
wt-infected mice	IFN γ	-13.96 \pm 0.18	-1.54 \pm 0.17	8.92 \pm 0.32	7.98 \pm 0.18
wt-naive mice	IP10	-2.89 \pm 0.20	1.66 \pm 0.24	2.54 \pm 0.25	5.35 \pm 0.22
wt-infected mice	IP10	-1.90 \pm 0.13	-4.32 \pm 0.14	-1.25 \pm 0.05	1.15 \pm 0.15
wt-naive mice	IIGP	-6.45 \pm 0.26	1.19 \pm 0.16	8.09 \pm 0.14	13.24 \pm 0.27
wt-infected mice	IIGP	-7.93 \pm 0.57	-4.53 \pm 0.12	3.61 \pm 0.22	2.30 \pm 0.11

^aData are expressed as means \pm SD in IFN γ -gut mice relative to naive or infected (48 h after oral infections with *Salmonella enterica* Typhimurium *aroA*⁻) wt mice. A negative value describes a lower response (lower mRNA level of IFN γ , IP10 or IIGP) in IFN γ -gut compared with wt mice. A positive value describes a higher response (higher mRNA level of IFN γ , IP10 or IIGP) in IFN γ -gut compared with wt mice.

expected, we did not observe any differences in $\text{IFN}\gamma$ message levels between infected and naive $\text{IFN}\gamma$ -gut mice as the $\text{Fabp1}^{4\times}$ at -132 promoter is not up-regulated upon infection (data not shown).

ELISA on systemic compartments such as the MLNs, liver and spleen did not reveal the presence of $\text{IFN}\gamma$ protein (data not shown). Similarly, we did not detect any $\text{IFN}\gamma$ in systemic compartments or in serum of $\text{IFN}\gamma$ -gut mice on western blots with antibodies against $\text{IFN}\gamma$ although they did bind to recombinant $\text{IFN}\gamma$ that was used as a positive control (data not shown).

Histological examination of the gut

$\text{IFN}\gamma$ -gut mice did not show any signs of infiltration, ulceration, mucosal thickening, haemorrhage or epithelial cell integrity (data not shown). Therefore, compared with the wt mice, $\text{IFN}\gamma$ -gut mice were free of apparent signs of pathology indicating that the levels of $\text{IFN}\gamma$ produced regionally in the $\text{IFN}\gamma$ -gut mice did not lead to chronic inflammation.

Oral infection with attenuated *S. enterica* Typhimurium

$\text{IFN}\gamma$ -gut, $\text{IFN}\gamma$ -KO and wt mice were orally infected with the wt, fully virulent *S. enterica* Typhimurium strain SL1344 (Fig. 1A) and CFU in spleen and MLN were determined. Using this strain, we observed similar CFU in MLNs and spleens of $\text{IFN}\gamma$ -KO and $\text{IFN}\gamma$ -gut mice. Once past the intestinal barrier, *S. enterica* Typhimurium rapidly multiplies, especially in the absence of $\text{IFN}\gamma$, making it difficult to determine any major difference in the CFU between wt, $\text{IFN}\gamma$ -KO and $\text{IFN}\gamma$ -gut mice. Therefore, mice were orally infected with the attenuated *aroA*⁻ *S. enterica* Typhimurium strain SL7207, which shows reduced growth *in vivo*. Figure 1(B) demonstrates that after SL7207 infection, the range of CFU in MLNs and spleens of $\text{IFN}\gamma$ -gut mice was similar to that in $\text{IFN}\gamma$ -KO mice, as well. Thus, regional $\text{IFN}\gamma$ expression in the distal part of the small intestine, caecum and colon was insufficient to control penetration of the gut epithelial barrier by salmonellae through uptake by PP, at least at the $\text{IFN}\gamma$ abundance produced by $\text{IFN}\gamma$ -gut mice.

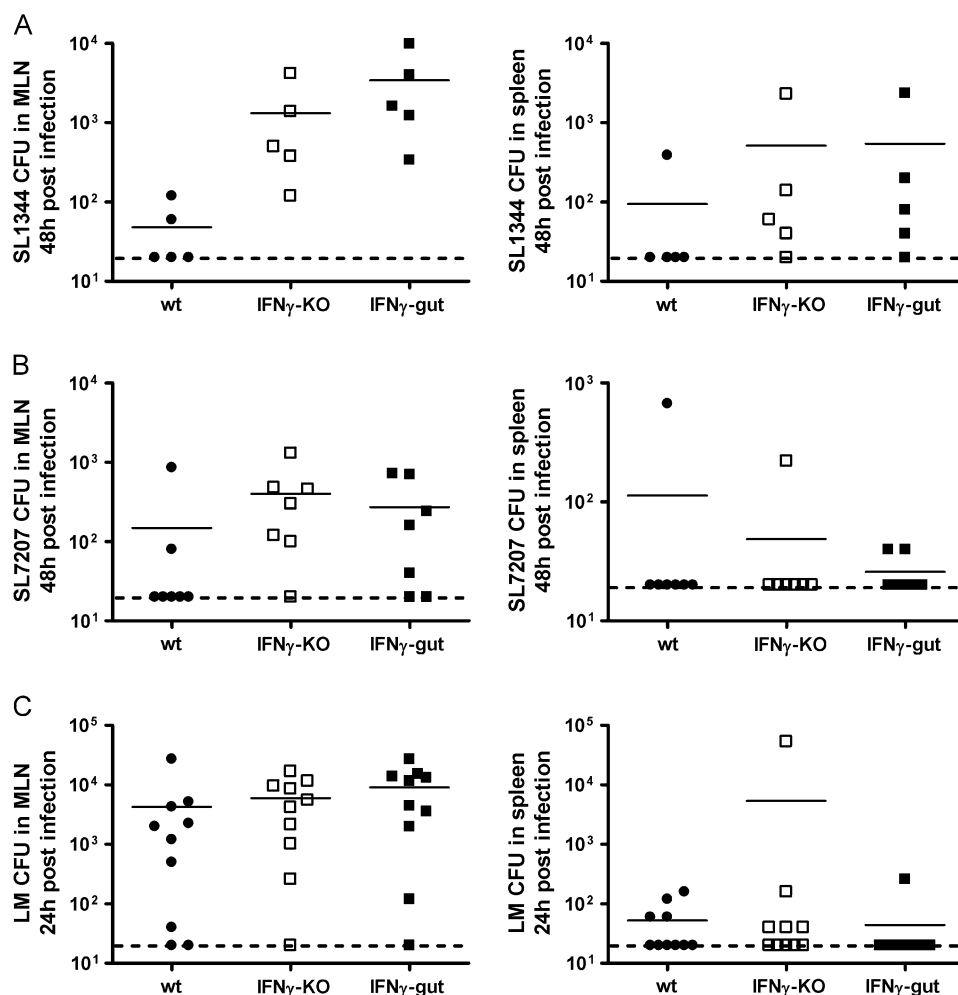


Fig. 1. Bacterial load in MLN and spleen 48 h after infections of wt, $\text{IFN}\gamma$ -gut and $\text{IFN}\gamma$ -KO mice. (A) Five mice per group were intragastrically infected with 1×10^8 wt *Salmonella enterica* Typhimurium SL1344. This experiment was repeated twice with similar results. (B) Seven mice per group were intragastrically infected with 1.8×10^9 *S. enterica* Typhimurium *aroA*⁻ strain. The experiment was repeated three times with similar results. (C) Bacterial load in MLN and spleen 24 h after intragastric infections of wt, $\text{IFN}\gamma$ -gut and $\text{IFN}\gamma$ -KO mice. Ten mice per group were infected with 3.0×10^9 *Listeria monocytogenes* EGD strain. This experiment was repeated twice with similar results.

Oral infection with *L. monocytogenes*

IFN γ -gut, IFN γ -KO and wt mice were orally infected with *L. monocytogenes*, and CFU in MLN and spleen were determined (Fig. 1C). Similar to *S. enterica* Typhimurium infection, listerial CFU in the MLN did not show significant differences among the three groups although CFU in MLN of wt mice were lower than those in IFN γ -KO mice. Hardly any CFU were detected in spleen suggesting the absence of significant injury during intragastric gavage. As with spleens, we

observed increased CFU in the liver after 48 h (data not shown). This was probably due to invasion and multiplication of bacteria rather than due to injury during intragastric gavage since in the latter case we would have already observed increased CFU after 24 h. Since the colon of IFN γ -gut mice expressed 44-fold more IFN γ compared with wt mice, and as the colon is drained through the CLN, CFU in CLN were determined after oral infection with *L. monocytogenes*. No significant differences were observed among the different groups of mice (data not shown). This may also be due to a narrow temporal window before listeriae invade the CLN.

Antigen-specific T cell responses in intestinal tissues

To determine whether local IFN γ expression in the gut affects antigen-specific CD4 $^{+}$ and CD8 $^{+}$ T cell responses, IFN γ -gut, IFN γ -KO and wt mice were orally infected with 2×10^9 CFU LmOVA. Bacteria were cleared by antibiotics at day 3 p.i. and LLO_{190–201}-specific CD4 $^{+}$ and OVA_{257–264}-specific CD8 $^{+}$ T cell responses were measured at day 9 p.i. in spleen, MLN and in the intraepithelial lymphocytes and LPL compartment (21, 22). Since IFN γ secretion by T cells could not be used for determination of specific T cells in IFN γ mutant mice, TNF α secretion was measured. Three independent experiments similarly demonstrated that the local expression of IFN γ affected neither LLO_{190–201}-specific CD4 $^{+}$ nor OVA_{257–264}-specific CD8 $^{+}$ T cell responses (Fig. 2). In contrast to wt mice, OVA-specific T cell responses were slightly increased in IFN γ -gut and in IFN γ -KO mice. This could be explained by the increased antigenic load in IFN γ -gut mice due to initially elevated bacterial titres or by the regulatory function of IFN γ , as recently described (24).

Discussion

The data presented suggest that exclusive IFN γ production in the gut is insufficient for control of penetration of the gut epithelial barrier by food-borne bacterial pathogens such as *S. enterica* Typhimurium and *L. monocytogenes*. Host invasion by listeriae after oral infection proceeds via the gastrointestinal tract (4). Because colon and rectum are drained by CLN (4), dissemination and subsequent systemic infection involve CLN. Penetration of the small intestine involves

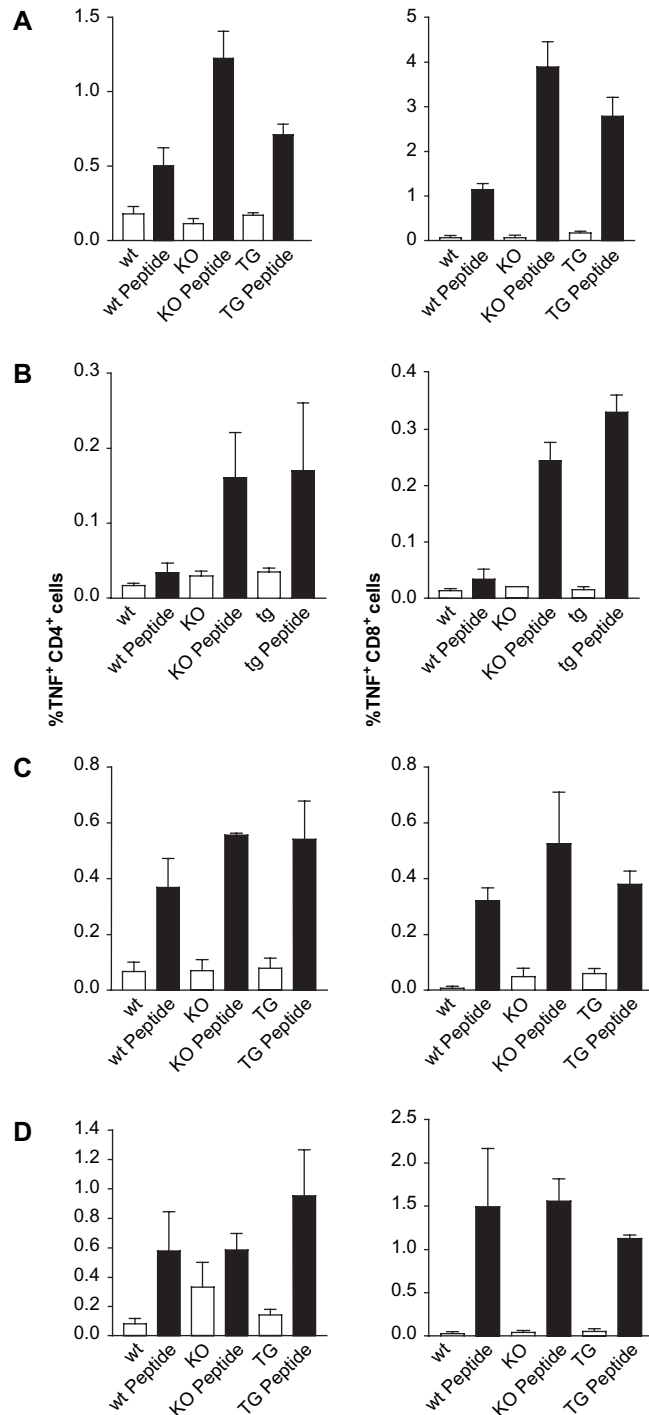


Fig. 2. Specific CD4 $^{+}$ and CD8 $^{+}$ T cell responses in intestinal tissues following infection of mice with *Listeria monocytogenes*. wt, IFN γ -KO (KO) and IFN γ -gut (tg) mice were intragastrically infected with 2.0×10^9 LmOVA. Nine days p.i., mice were killed and lymphocytes were isolated from spleen (A), MLNs (B), the lamina propria (C) and the epithelium (D) of the small intestine. Cells were incubated for 5 h without peptide or with the peptides LLO_{190–201} or OVA_{257–264} to stimulate listeriolytic-specific CD4 $^{+}$ T cells or OVA-specific CD8 $^{+}$ T cells, respectively. Following incubation, cells were stained extracellularly with anti-CD4 mAb or anti-CD8 α mAb and intracellularly with anti-TNF α mAb and analysed by flow cytometry. Bars in figures show frequencies of TNF α -secreting cells among CD4 $^{+}$ (left column) or CD8 $^{+}$ T cells (right column) following incubation with (black bars) and without peptide (white bars). Bars represent mean \pm SD of three individually analysed mice. The experiment shown is representative of three independent experiments, each with three individually analysed mice per mouse strain.

uptake by PP from where listeriae spread via the lymph to the spleen and liver. Salmonellae, on the other hand, penetrate the gut epithelial barrier mainly through the PP in the small intestine. General failure to produce IFN γ increases susceptibility of mice to bacterial infection (3). Thus, evidence has been presented that IFN γ produced in the intestine plays a role in regional host defence against food-borne bacterial pathogens (3, 15). In this study, we aimed at determining the impact of gut-specific IFN γ expression on regional host defence against food-borne bacterial infection. To achieve this goal, we created IFN γ -gut transgenic mice on an IFN γ -KO background, selectively expressing IFN γ in the small intestine, caecum and colon.

The IFN γ -gut mice expressed an increasing gradient of IFN γ from the distal region of the small intestine to the colon (see Table 1). In the distal part of the small intestine, the amount of IFN γ mRNA was comparable to naive wt controls and towards the proximal region it was lower than in wt mice. IFN γ -gut caecum and colon had 19-fold and 44-fold more IFN γ mRNA, respectively, relative to wt mice. These levels are not reached during an oral infection of wt mice with *S. enterica* Typhimurium *aroA*⁻. This became apparent when comparing caecum and colon of IFN γ -gut mice relative to infected wt mice, where the levels of IFN γ -gut were only 9-fold to 8-fold higher. The IFN γ -responsive genes, *IP10* and *IIGP*, also followed this pattern in the caecum and colon, i.e. higher IFN γ expression resulted in more *IP10* and *IIGP* mRNA expression, demonstrating that the IFN γ expressed in IFN γ -gut mice was functional. In addition, minute expression was found in the kidney but none in liver and stomach (data not shown) as expected from the Fabp1^{4 \times} at -132 promoter expression pattern. This promoter was reported to drive expression in goblet, enteroendocrine, and Paneth cells throughout the duodenal-ileal axis and in colon that lacks Paneth cells (20).

Our data reveal that regional IFN γ production in the intestine (at least at the moderate levels in the small intestine with an increasing gradient towards the colon, produced in the IFN γ -gut mice described here) is insufficient for control of the spread of salmonellae and listeriae from the gut to the MLN. Because salmonellae penetrate the gut epithelial barrier mainly through uptake by PP, this gradient of IFN γ expression may explain the lower *Salmonella* burden in MLN of IFN γ -gut mice as compared with IFN γ -KO mice and higher *Salmonella* burden as compared with wt mice. We assume that in the proximal part of the small intestine, little or virtually no IFN γ is produced to activate antibacterial capacities in macrophages. In this region, salmonellae can penetrate the epithelium through PP. Towards the distal region of the small intestine, IFN γ expression increased and could partly contribute to control of the salmonellae resulting in lower CFU in MLN. Although in IFN γ -gut mice IFN γ was not expressed in PP, we cannot exclude that IFN γ may have diffused into the PP and stimulated immune cells. However, in contrast to the extent of control of salmonellae seen in wt mice, lack of sufficient control of salmonellae in IFN γ -gut mice suggests that the amount of IFN γ produced by gut epithelial cells that may have entered the PP was too low to compensate for lack of IFN γ production by immune cells in PP. Another possibility is that the immune response mounted against salmonellae in the distal part of

the small intestine in IFN γ -gut mice was more efficacious due to IFN γ expression whereas in the proximal part and in the other parts of the small intestine, immunity was weak or absent. This could explain apparent lack of an effective immune response in MLN of IFN γ -gut mice. Thus, in spite of local IFN γ expression, no significant differences were observed between IFN γ -gut and IFN γ -KO mice in host defence against oral *Salmonella* and *Listeria* infections, although after oral *Salmonella* infection the CFU in MLN of IFN γ -gut mice were lower than those in IFN γ -KO mice. As expected, wt mice showed lower CFU in MLN compared with IFN γ -KO mice when infected with either salmonellae or listeriae.

It could be argued that the IFN γ concentration in IFN γ -gut mice was below the required threshold levels for macrophage activation. However, real-time PCR data suggest that the lowest levels of IFN γ that were detected in the distal part of the small intestine in IFN γ -gut mice were similar to IFN γ concentrations measured in naive wt mice and increased up to 44-fold, relative to wt mice, in the colon. Yet, CFU in IFN γ -gut mice were consistently higher than those in the caecum and colon of wt mice. Given that regional IFN γ expression in the gut was sufficient for control at the gut barrier, the CFU in MLN of IFN γ -gut mice should have been significantly less than CFU in MLN of IFN γ -KO mice, at least with listeriae, which can penetrate the gut epithelial barrier through PP-independent mechanisms.

There is some controversy as to which immune cells produce IFN γ during bacterial infection. NK cells, T cells, DCs and macrophages are thought to be major producers of IFN γ although it has been reported that IFN γ produced by macrophages and DCs rather than by NK cells and T cells primarily contributes to control of listerial infections, after intra-peritoneal administration (16). In any case, our results imply that immune cells, rather than gut epithelial cells, represent the crucial source of IFN γ in defence against food-borne bacterial infections.

Because NK cells, T cells, DCs and macrophages in IFN γ -gut mice do not produce IFN γ (due to the general IFN γ deficiency), we assume that IFN γ production by these immune cells is essential for effective antibacterial defence against salmonellae and listeriae at the gut barrier in normal mice. In these mice, IFN γ produced by NK cells and T cells during infection probably stimulates additional IFN γ secretion in antigen-presenting cells namely macrophages and DCs (16). This could ultimately cause efficient activation of antibacterial capacities in macrophages. This feedback loop is interrupted in IFN γ -gut and IFN γ -KO mice because of general IFN γ deficiency. It has to be noted that LLO₁₉₀₋₂₀₁-specific CD4⁺ and OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell functions were not impaired in both mutant mouse strains. In contrast, as a consequence of the reduced anti-microbial capacity of IFN γ mutant mice, the increased antigenic load in these mice may explain slightly enhanced frequencies of antigen-specific CD4⁺ and CD8⁺ T cells as compared with wt mice.

In conclusion, our experiments suggest that regional IFN γ expression by non-immunological cells at the gut barrier is insufficient for regional defence against food-borne infection with *S. enterica* and *L. monocytogenes*. On the other hand, in wt mice, IFN γ clearly plays a central role in the defence against these pathogens and it is possible that increasing

the amount of regional IFN γ expression in the gut improves control of bacterial infections at the gut barrier. Such constitutively produced high IFN γ levels, however, could favour the development of chronic inflammation (25).

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Abbreviations

CFU	colony-forming units
CLN	caudal lymph node
Fabpl	liver fatty acid-binding protein
IIGP	IFN-inducible GTPase
IP10	IFN γ -inducible protein-10
KO	knock-out
LB	Luria-Bertani
LLO _{190–201}	listeriolysin O amino acids 190–201
LmOVA	<i>Listeria monocytogenes</i> strain-expressing ovalbumin
MLN	mesenteric lymph node
OVA _{257–264}	ovalbumin _{257–264}
p.i.	post-infection
PP	Peyer's patches
TNF α	tumour necrosis factor α
wt	wild type

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